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(54) Title: DIAGNOSIS AND TREATMENT OF DISEASES ARISING FROM DEFECTS IN THE TUBEROUS SCLEROSIS PATHWAY

(57) Abstract: The present invention relates to compositions and methods for identifying abnormalities in TSC signaling pathways. In particular, the present invention relates to methods of diagnosing and treating disorders such as tuberous sclerosis, which are caused by mutations in the TSC genes. The present invention further relates to methods and compositions for treating cancers mediated by TSC signaling disorders.

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DIAGNOSIS AND TREATMENT OF DISEASES ARISING FROM DEFECTS IN THE TUBEROUS SCLEROSIS PATHWAY

The present application claims priority to U.S. Provisional Application Serial Number 60/402,718, filed August 12, 2002, herein incorporated by reference.

This invention was made with government support under Grant No GM51586 awarded by the National Institutes of Health. The Government has certain rights in the invention.

FIELD OF THE INVENTION

The present invention relates to compositions and methods for identifying abnormalities in TSC signaling pathways. In particular, the present invention relates to methods of diagnosing and treating disorders such as tuberous sclerosis, which are caused by mutations in the TSC genes. The present invention further relates to methods and compositions for treating cancers mediated by TSC signaling disorders.

BACKGROUND

Tuberous Sclerosis (TSC) is a relatively common inheritable genetic disorder that occurs in approximately 1 in 6000 of the population and is characterized by the development of hamartomas in a variety of organs (Young & Povey, *Mol. Med. Today* 4, 313–319 (1998)). Common clinical symptoms include seizures, mental retardation, autism, kidney failure, facial angiofibromas and cardial Rhabdomyomas (Gomez, *Ann. NY Acad. Sci.* **615**, 1–7 (1991)). In addition, many affected individuals have cyst-like areas within certain skeletal regions, particularly bones of the fingers and toes (phalanges). Characteristic skin lesions include sharply defined areas of decreased skin coloration (hypopigmentation) that may develop during infancy and relatively small reddish nodules that may appear on the cheeks and nose beginning at approximately age four. These reddish lesions eventually enlarge, blend together (coalesce), and develop a wart-like appearance (sebaceous adenomas). Additional skin lesions may also develop, including flat, "coffee-colored" areas of increased skin pigmentation (café-au-lait spots); benign, fibrous nodules (fibromas) arising around or beneath the nails; or rough, elevated, "knobby" lesions (shagreen patches) on the lower back.

TSC may be present at birth, but signs of the disorder can be subtle and full symptoms may take some time to develop. As a result, TSC is frequently unrecognized and

misdiagnosed for years. In most cases the first clue to recognizing TSC is the presence of seizures or delayed development. In other cases, the first sign may be white patches on the skin (hypomelanotic macules).

Diagnosis of the disorder is based on a careful clinical exam in combination with computed tomography (CT) or magnetic resonance imaging (MRI) of the brain, which may show tubers in the brain, and an ultrasound of the heart, liver, and kidneys, which may show tumors in those organs. Diagnosis also involves a careful examination of the skin for the wide variety of skin features, the fingernails and toenails for ungual fibromas, the teeth and gums for dental pits and/or gum fibromas, and the eyes for dilated pupils. A Wood's lamp or ultraviolet light may be used to locate the hypomelantic macules, which are sometimes hard to see on infants and individuals with pale or fair skin.

In infants TSC may be suspected if the child has cardiac rhabdomyomas or seizures (infantile spasms) at birth. With a careful examination of the skin and brain, it may be possible to diagnose TSC in a very young infant. However, most children are not diagnosed until later in life when their seizures begin and other symptoms such as facial angiofibromas appear.

There is no specific treatment for tuberous sclerosis. Treatment is symptomatic and may include anticonvulsant therapy for seizures, dermabrasion and laser removal techniques for the skin manifestations, drug therapy for neurobehavioral problems, treatment of high blood pressure caused by the kidney problems, and surgery to remove growing tumors.

The prognosis for individuals with tuberous sclerosis varies depending on the severity of symptoms. There is no cure. Those individuals with mild symptoms generally do well and live long productive lives, while individuals with the more severe form may have serious disabilities. In rare cases, seizures, infections, or tumors in vital organs may cause complications in some organs such as the kidneys and brain that can lead to severe difficulties and even death.

Improved TSC early diagnostics are needed to allow for earlier treatment. Additional therapeutics are also needed. Preferred therapeutics are those that treat symptoms systemically.

SUMMARY OF THE INVENTION

The present invention relates to compositions and methods for identifying abnormalities in TSC signaling pathways. In particular, the present invention relates to methods of diagnosing and treating disorders such as tuberous sclerosis, which are caused

by mutations in the TSC genes. The present invention further relates to methods and compositions for treating cancers mediated by TSC signaling disorders.

Accordingly, in some embodiments, the present invention provides a method of detecting increased S6 kinase activity in a subject, comprising providing a biological sample from a subject; and detecting increased S6 kinase activity in the biological sample. In some embodiments, detecting increased S6 kinase activity comprises a S6 kinase phosphatase assay. For example, in some embodiments, the S6 kinase phosphatase assay comprises hybridizing a phosphospecific antibody to a S6 kinase substrate. In certain embodiments, increased S6 kinase activity is indicative of an inactivated protein selected from the group consisting of TSC1 protein and TSC2 protein. In some embodiments, the inactivated protein is due to a mutation (*e.g.*, a truncation) in a gene encoding said TSC1 protein or said TSC2 protein. In some embodiments, the present invention further comprises the step of providing a diagnosis to the subject based on said detecting increased S6 kinase activity. In some embodiments, the diagnosis is a diagnosis of tuberous sclerosis in said subject. In some embodiments, the present invention further comprises the step of providing treatment for tuberous sclerosis to said subject. In some embodiments, the treatment comprises administering a S6 kinase inhibitor to said subject. The present invention is not limited to a particular S6 kinase inhibitor. Any suitable S6 kinase inhibitor is contemplated including, but not limited to, rapamycin and rapamycin derivatives.

The present invention also provides a kit for the diagnosis of tuberous sclerosis, comprising reagents for detecting increased S6 kinase activity in a subject. In some embodiments, the reagents comprise a phosphospecific antibody specific for an S6 kinase substrate. In some embodiments, the kit further comprises instruction for using the reagents for diagnosing tuberous sclerosis in the subject. In certain embodiments, the instructions comprise instructions required by the United States Food and Drug Administration for use in *in vitro* diagnostic products.

The present invention further provides a method of treating tuberous sclerosis in a subject, comprising providing a subject diagnosed with tuberous sclerosis; and an inhibitor of S6 kinase; and administering the inhibitor to the subject. In some preferred embodiments, the administering results in a decrease in symptoms of tuberous sclerosis in the subject. The present invention is not limited to a particular S6 kinase inhibitor. Any suitable S6 kinase inhibitor is contemplated including, but not limited to, rapamycin and rapamycin derivatives.

In yet other embodiments, the present invention provides a method of screening compounds, comprising providing a cell expressing S6 kinase; and one or more test compounds; and screening the test compounds for the ability to inhibit the kinase activity of said S6 kinase. In some embodiments, screening the compounds for the ability to inhibit the kinase activity of S6 kinase activity comprises a S6 kinase phosphatase assay. In some embodiments, the S6 kinase phosphatase assay comprises hybridizing a phosphospecific antibody to a S6 kinase substrate. In some embodiments, the cell is *in vitro*. In some embodiments, the cell is a TSC2-/- cell. In other embodiments, the cell is *in vivo*. In some embodiments, the cell is in a non-human animal (*e.g.*, a rat or a mouse). In some embodiments, the rat is an Eker rat. In some embodiments, the test compound is a drug. In some embodiments, the test compound is rapamycin. In other embodiments, the test compound is a derivative of rapamycin. The present invention further provides a drug identified by the method.

In other embodiments, the present invention provides a method of treating a disease, comprising providing a subject suffering from a disease, and an agent capable of reducing cellular energy levels, and administering the agent to the subject. In preferred embodiments, the disease comprises defective cells. In further embodiments, the defective cells comprise a defective TSC pathway. In even further embodiments, the method further provides co-administering rapamycin to the subject.

In preferred embodiments, the defective TSC pathway comprises a defective element of the TSC pathway such as TSC1, TSC2, Rheb, mTOR, S6K, and/or 4EBP-1.

In some preferred embodiments, the agent targets the defective cells. In other embodiments, the agent inhibits hexokinase. In other embodiments, the agent is 2-deoxy-glucose. In other embodiments, the agent is the mitochondrial uncoupler FCCP. In other embodiments, the agent inhibits PKC. In other embodiments, the agent is Rottlerin. In even other embodiments, the agent is 5-aminoimidazole-4-carboxyamide ribonucleotide.

In other preferred embodiments, the disease is tuberous sclerosis. In other embodiments, the disease is cancer.

DESCRIPTION OF THE FIGURES

Figure 1 shows inhibition of S6K by TSC1–TSC2. Figure 1a shows that TSC1–TSC2 inhibits S6K kinase activity. HA–S6K was transfected in HEK293 cells in the presence or absence of TSC1–TSC2, as indicated. Figure 1b shows that TSC1–TSC2 selectively inhibits phosphorylation of Thr 389, but not Thr 421/Ser 424 of S6K. Figure 1c

shows that TSC1–TSC2 does not inhibit Ras-induced activation of ERK. Figure 1d shows the dose dependent inhibition of S6K phosphorylation on Thr 389 (left). TSC1–TSC2 has no effect on either basal or insulin-stimulated phosphorylation of Akt (right).

Figure 2 shows the effects of endogenous TSC2 and disease-derived mutations on phosphorylation of S6K. Figure 2a shows enhancement of basal and stimulated phosphorylation of S6K by *TSC2* RNA interference. Figure 2b shows increased phosphorylation of endogenous S6K and S6 by *TSC2* RNA interference. Figure 2c shows that disease-derived TSC2 mutants are compromised in their ability to inhibit S6K.

Figure 3 shows phosphorylation of TSC2 by Akt. Figure 3a shows the Akt-dependent mobility shift of TSC2. Figure 3b shows two-dimensional phosphopeptide mapping of *in vivo* ^{32}P-labelled TSC2. Figure 3c shows two-dimensional phosphopeptide mapping of HA–TSC2 in the presence of insulin (400 nM), LY294002 (50 μM) or rapamycin (20 nM).

Figure 4 shows the determination of Akt-dependent phosphorylation sites in TSC2. Figure 4a shows a schematic representation of putative Akt phosphorylation sites in TSC2. The sites conserved in *Drosophila* dTsc2 are boxed. The TSC2 fragment 1 and fragment 2 regions used for the *in vitro* Akt phosphorylation assay are indicated. Figure 4b shows mutational analysis of Akt phosphorylation sites. Mutants are indicated below each panel. Panel VIII is a schematic representation of the boxed region, denoting the specific phosphorylation sites altered by the corresponding alanine substitutions. Phosphopeptides that are missing in each mutant are indicated by open circles in panels I, II, IV and VI. Figure 4c shows phosphorylation of recombinant TSC2 fragments by purified Akt. Two-dimensional phosphopeptide mapping of the *in-vitro*-phosphorylated TSC2 fragments is also shown (bottom).

Figure 5 shows that mutation of Akt phosphorylation sites alters TSC2 activity. Figure 5a shows that substitution of phosphorylation sites by alanine increases TSC2 activity, whereas substitution with acidic residues decreases activity. Figure 5b shows inhibition of 4E-BP1 phosphorylation by TSC2 mutants. Figure 5c shows that acidic residue substitutions disrupt formation of the TSC1–TSC2 complex. Figure 5d shows that the phosphomimetic mutant of TSC2 is unstable. The stability of TSC2 was determined in the presence of cycloheximide (300 μM, 0–6 h). Figure 5e shows that the phosphomimetic TSC2 mutant is highly ubiquitinated.

Figure 7 shows that TSC1–TSC2 functions through mTOR to inhibit S6K. Figure 7a shows that the phosphorylation of Thr 389 of the S6K-dC104 mutant is not inhibited by

rapamycin. Figure 7b shows that TSC1–TSC2 does not inhibit Thr 389 phosphorylation of the S6K-dC104 mutant. Figure 7c shows that TSC1–TSC2 does not inhibit insulin-induced S6K-dNC kinase activity. Figure 7d shows that TSC1–TSC2 inhibits mTOR kinase activity. Figure 7e shows that TSC1–TSC2 inhibits phosphorylation of mTOR. Cotransfection of TSC1–TSC2 inhibits phosphorylation of Ser 2448 on mTOR, as detected by immunoblotting with an anti-phospho-mTOR antibody (left). A reduction of endogenous TSC2 by RNAi-C increased phosphorylation of mTOR (right). Figure 7f shows a proposed model for TSC1–TSC2 function in the regulation of cell growth.

Figure 8 shows the effects of energy depletion on phosphorylation of S6K, S6, 4EBP1, mTOR, Akt, AMPK, and TSC2 by 2-DG. Figure 8a shows dephosphorylation of S6K and 4EBP1 by ATP depletion. Figure 8b shows mobility shift of TSC2. Figure 8c shows 2-DG-induces dephosphorylation of endogenous S6K, S6, 4EBP1, mTOR but not AKT. Figure 8d shows 2-DG-induces phosphorylation of AMPK. Figure 8e shows time course of 2-DG treatment. Figure 8f shows 2-DG decreases intracellular ATP levels. Figure 8g shows 2-DG increases the AMP/ATP ratio. Figure 8h shows low glucose inhibits S6K.

Figure 9 shows 2-DG stimulates the interaction of endogenous AMPK and TSC2. Figure 9a shows 2-DG stimulates the co-immunoprecipitation between endogenous TSC2 and AMPK. Figure 9b shows reciprocal immunoprecipitation of endogenous TSC2 and AMPK. Figure 9c shows expression levels of endogenous TSC1, TSC2, pAMPK and AMPK in HEK293 cells. Figure 9d shows the C-terminal fragment of TSC2 interacts with endogenous AMPK.

Figure 10 shows TSC2 is required for 2-DG induced dephosphorylation of S6K. Figure 10a shows knockdown of TSC2 by RNA interference blocks the 2-DG response. Figure 10b shows knockdown of TSC2 by RNA interference does not block rapamycin-induced dephosphorylation of S6K. Figure 10c shows inhibition of S6K by AMPK overexpression is blocked by TSC2 RNAi. Figure 10d shows knockdown of TSC2 has little effect on the 2-DG-induced phosphorylation of ACC and eEF2. Figure 10e shows rapamycin has little effect on the 2-DG-induced phosphorylation of ACC and eEF2. Figure 10f shows ATP depletion-induced dephosphorylation of S6K and 4EBP1 are compromised in TSC2 -/- cells. Figure 10g shows 2-DG induced 4EBP1 dephosphorylation is compromised in TSC2 -/- cells.

Figure 11 shows ATP depletion and AMPK induce TSC2 phosphorylation. Figure 11a shows 2-DG induced TSC2 mobility shift is due to phosphorylation. Figure 11b shows

AMPK expression induces a slow migrating form of TSC2. HA-TSC1 and Myc-TSC2 were co-transfected with or without active AMPK αI subunit as indicated and blotted by anti-HA and anti-Myc antibodies, respectively. Figure 11c shows an AMPK inhibitor blocks the 2-DG induced mobility shift of TSC2. AMPK inhibitor (Compound C, 10 μM) was added 30 minutes before the treatment of 2-DG as indicated. Figure 11d shows kinase inactive AMPK mutant blocks 2-DG-induced dephosphorylation of S6K. HEK293 cells were transfected with increasing amounts of the kinase inactive AMPK mutant (AMPKDN). Figure 11e shows AMPK inhibitor blocks the 2-DG induced dephosphorylation of S6K. Figure 11f shows AMPK inhibitor partially blocks S6K dephosphorylation induced by glucose deprivation.

Figure 12 shows AMPK phosphorylates TSC2 on S1227 and S1345. Figure 12a shows 2-DG and AMPK induce TSC2 phosphorylation on multiple spots *in vivo*. Figure 12b shows S1337 and S1341 are AMPK-dependent sites phosphorylated by 2-DG treatment. Figure 12c shows AMPK directly phosphorylates TSC2 on S1345 but not S1337 or 1341 *in vitro*. Figure 12d shows Ser1345 in TSC2 is phosphorylated *in vivo*. Figure 12e shows T1227 in TSC2 is phosphorylated *in vivo*. Figure 12f shows AMPK phosphorylates TSC2 on T1227 *in vitro*. Figure 12g shows wild type TSC2 but not the S1337A/S1341A/S1345A mutant shows a mobility shift in response to 2-DG.

Figure 13 shows AMPK phosphorylation is important for TSC2 function in the regulation of S6K phosphorylation in response to energy limitation. Figure 13a shows mutation of the AMPK-dependent sites in TSC2 decreases TSC2 activity. Figure 13b shows mutant TSC2 can form complex with TSC1. Figure 13c shows cells expressing the AMPK phosphorylation mutant TSC2 (T1227A/S1345A) are less responsive to 2-DG treatment. Figure 13d shows TSC2-3A mutant is less active to inhibit S6K. LEF (TSC2-/- epithelial) cells were infected with TSC2 retrovirus and selected for the neomycin resistant stable expressing cells. Figure 13e shows the AMPK-dependent phosphorylation of TSC2 is important for glucose deprivation-induced S6K dephosphorylation.

Figure 14 shows TSC2 plays essential roles in protecting cells from glucose deprivation-induced apoptosis and cell size regulation. Figure 14a shows TSC2 but not TSC2-3A protects LEF cells from glucose deprivation-induced cell death. Figure 14b shows glucose deprivation induces DNA fragmentation in vector and TSC2-3A but not in TSC2 expressing LEF cells. Figure 14c shows glucose deprivation induces cleavage of caspase-3 and PARP in vector and TSC2-3A but not in TSC2 expressing LEF cells. Figure 14d shows 2-DG decreases cell size in HEK293 cells. HEK293 cells were cultured in the

presence of 12.5 mM 2-DG, TSC2 RNAi, or 20 nM rapamycin for 72 hours. Figure 14e shows low glucose (2.8 mM) decreases cell size in HEK293 cells. Figure 14f shows TSC2-3A is defective in cell size regulation. Figure 14g shows a proposed model of TSC2 in cellular energy signaling pathway.

DEFINITIONS

To facilitate an understanding of the present invention, a number of terms and phrases are defined below:

As used herein, the terms "S6K" and "S6 kinase" are used interchangeably to refer to an S6K kinase (e.g., the human or non-human S6K kinase).

As used herein, the term "detecting increased S6 kinase activity in said biological sample" refers to detecting, using any suitable method, the presence of increased kinase activity of an S6 kinase relative to the level of kinase activity of a control sample (*e.g.*, a control sample obtained from an individual known to have a normal level of S6 kinase activity). In some embodiments, kinase activity is assayed using the immunoassay described in the experimental section below. However, any assay that is capable of providing measure of kinase activity relative to a control may be utilized. In some embodiments, increased S6 kinase activity is indicative of an inactive TSC1 or TSC2 protein.

As used herein, the term "positive diagnosis of tuberous sclerosis in said subject" refers to a diagnosis of tuberous sclerosis in a subject. As used herein, the term "negative diagnosis of tuberous sclerosis in said subject" refers to the diagnosis of a subject of not having tuberous sclerosis.

As used herein, the term "TSC pathway" or "tuberous sclerosis complex pathway" refers generally to biological (*e.g.*, molecular, genetic, cellular, biochemical, pharmaceutical, environmental) events (*e.g.*, cellular pathways, cellular mechanisms, cellular cascades) involving the TSC-1 gene, the TSC-1 protein, the TSC-2 gene, and / or the TSC-2 protein. Examples of components of the TSC pathway include, but are not limited to, TSC-1, TSC-2, TSC-1/TSC-2, Rheb, mTOR, S6K, and 4EBP-1.

As used herein, the term "a subject with tuberous sclerosis" refers generally to a subject who has a defective TSC pathway. A defective TSC pathway may be identified by any recognized identification method (*e.g.*, phenotypically, genetically, biochemically, and molecularly). One method for identifying subjects with tuberous sclerosis involves

administration of a diagnostic assay to detect a defective TSC pathway (*e.g.*, the diagnostic assay tests described heren).

As used herein, the term "a subject diagnosed with tuberous sclerosis" refers to a subject that has been medically determined (*e.g.*, by a treating physician) as having tuberous sclerosis.

As used herein, the term "defective TSC pathway" or "sample having a defective TSC pathway" refers to samples demonstrated to have dysregulation (*e.g.*, regulation of the pathway that results in a biological effect that causes adverse effects on a cell or tissue) within the TSC pathway (*e.g.*, phenotypically, genetically, biochemically, and molecularly). One method of identifying a defective TSC pathway involves administration of a diagnostic assay to detect a defective TSC pathway (*e.g.*,the diagnostic assay tests described herein).

As used herein, the term "reduces cellular energy levels" or "reduction of cellular energy levels" refers generally to a reduction (*e.g.*, lowering, diminishing, lessening) of cellular glucose levels, amino acid levels, or ATP levels.

As used herein the term "said agent reduces cellular energy levels" or "methods of reducing cellular energy levels" refer generally to a targeting of cellular energy. Examples include, but are not limited to ATP and glucose. In addition, the term also refers generally to a targeting of components that assist in generating cellular energy. Examples include, but are not limited to, mitochondria, enzymes used to generate ATP (*e.g.*, hexokinase), energy generating pathways (*e.g.*, Krebs cycle), and drugs that regulate ATP metabolism (*e.g.*, 2-deoxy-glucose).

As used herein, the term "hypertrophy" generally refers to the enlargement or overgrowth of an organ or body part due to an increase in size of its constituent cells. Examples include, but are not limited to, right ventricular hypertrophy, hypertrophic cardiomyopathy, and benign prostatic hypertrophy.

As used herein, the term "S6 kinase inhibitor" refers to a compound that inhibits the kinase activity of S6 kinase. In preferred embodiments, inhibitors inhibit the kinase activity to the level of kinase activity seen in a control sample. In particularly preferred embodiments, S6 kinase inhibitors reduce symptoms of diseases caused by increased S6 kinase activity (*e.g.*, tuberous sclerosis).

The term "epitope" as used herein refers to that portion of an antigen that makes contact with a particular antibody.

When a protein or fragment of a protein is used to immunize a host animal, numerous regions of the protein may induce the production of antibodies which bind

specifically to a given region or three-dimensional structure on the protein; these regions or structures are referred to as "antigenic determinants". An antigenic determinant may compete with the intact antigen (*i.e.*, the "immunogen" used to elicit the immune response) for binding to an antibody.

The terms "specific binding" or "specifically binding" when used in reference to the interaction of an antibody and a protein or peptide means that the interaction is dependent upon the presence of a particular structure (*i.e.*, the antigenic determinant or epitope) on the protein; in other words the antibody is recognizing and binding to a specific protein structure rather than to proteins in general. For example, if an antibody is specific for epitope "A," the presence of a protein containing epitope A (or free, unlabelled A) in a reaction containing labeled "A" and the antibody will reduce the amount of labeled A bound to the antibody.

As used herein, the terms "non-specific binding" and "background binding" when used in reference to the interaction of an antibody and a protein or peptide refer to an interaction that is not dependent on the presence of a particular structure (*i.e.*, the antibody is binding to proteins in general rather that a particular structure such as an epitope).

As used herein, the term "subject" refers to any animal (*e.g.*, a mammal), including, but not limited to, humans, non-human primates, rodents, and the like, which is to be the recipient of a particular treatment. Typically, the terms "subject" and "patient" are used interchangeably herein in reference to a human subject.

As used herein, the term "phosphospecific antibody" refers to an antibody that specifically binds to the phosphorylated form of a polypeptide (*e.g.*, S6K) but does not specifically bind to the non-phosphorylated form of a polypeptide. In some embodiments, phosphospecific antibodies specifically bind to a polypeptide phoshphorylated at a specific position.

As used herein, the term "instructions for using said kit for detecting tuberous sclerosis in said subject" includes instructions for using the reagents contained in the kit for the detection and/or characterization of tuberous sclerosis in a biological sample from a subject. In some embodiments, the instructions further comprise the statement of intended use required by the U.S. Food and Drug Administration (FDA) in labeling analyte specific reagents (ASRs) or *in vitro* diagnostic products. The FDA classifies *in vitro* diagnostics as medical devices and requires that they be approved through the 510(k) procedure. Information required in an application under 510(k) includes: 1) The *in vitro* diagnostic product name, including the trade or proprietary name, the common or usual name, and the

classification name of the device; 2) The intended use of the product; 3) The establishment registration number, if applicable, of the owner or operator submitting the 510(k) submission; the class in which the *in vitro* diagnostic product was placed under section 513 of the FD&C Act, if known, its appropriate panel, or, if the owner or operator determines that the device has not been classified under such section, a statement of that determination and the basis for the determination that the *in vitro* diagnostic product is not so classified; 4)Proposed labels, labeling and advertisements sufficient to describe the *in vitro* diagnostic product, its intended use, and directions for use. Where applicable, photographs or engineering drawings should be supplied; 5) A statement indicating that the device is similar to and/or different from other *in vitro* diagnostic products of comparable type in commercial distribution in the U.S., accompanied by data to support the statement; 6) A 510(k) summary of the safety and effectiveness data upon which the substantial equivalence determination is based; or a statement that the 510(k) safety and effectiveness information supporting the FDA finding of substantial equivalence will be made available to any person within 30 days of a written request; 7) A statement that the submitter believes, to the best of their knowledge, that all data and information submitted in the premarket notification are truthful and accurate and that no material fact has been omitted; 8) Any additional information regarding the *in vitro* diagnostic product requested that is necessary for the FDA to make a substantial equivalency determination. Additional information is available at the Internet web page of the U.S. FDA.

As used herein, the terms "computer memory" and "computer memory device" refer to any storage media readable by a computer processor. Examples of computer memory include, but are not limited to, RAM, ROM, computer chips, digital video disc (DVDs), compact discs (CDs), hard disk drives (HDD), and magnetic tape.

As used herein, the term "providing a diagnosis to said subject based on said detecting increased S6 kinase activity" refers to providing a medical diagnosis (*e.g.*, of tuberous sclerosis) based on the presence of increased S6 kinase activity in the subject.

As used herein, the term "computer readable medium" refers to any device or system for storing and providing information (*e.g.*, data and instructions) to a computer processor. Examples of computer readable media include, but are not limited to, DVDs, CDs, hard disk drives, magnetic tape and servers for streaming media over networks.

As used herein, the terms "processor" and "central processing unit" or "CPU" are used interchangeably and refer to a device that is able to read a program from a computer

memory (*e.g.*, ROM or other computer memory) and perform a set of steps according to the program.

As used herein, the term "non-human animals" refers to all non-human animals including, but are not limited to, vertebrates such as rodents, non-human primates, ovines, bovines, ruminants, lagomorphs, porcines, caprines, equines, canines, felines, aves, etc.

As used herein, the term "nucleic acid molecule" refers to any nucleic acid containing molecule, including but not limited to, DNA or RNA. The term encompasses sequences that include any of the known base analogs of DNA and RNA including, but not limited to, 4-acetylcytosine, 8-hydroxy-N6-methyladenosine, aziridinylcytosine, pseudoisocytosine, 5-(carboxyhydroxylmethyl) uracil, 5-fluorouracil, 5-bromouracil, 5-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyluracil, dihydrouracil, inosine, N6-isopentenyladenine, 1-methyladenine, 1-methylpseudouracil, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-methyladenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarbonylmethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, oxybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, N-uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, pseudouracil, queosine, 2-thiocytosine, and 2,6-diaminopurine.

The term "gene" refers to a nucleic acid (*e.g.*, DNA) sequence that comprises coding sequences necessary for the production of a polypeptide, precursor, or RNA (*e.g.*, rRNA, tRNA). The polypeptide can be encoded by a full length coding sequence or by any portion of the coding sequence so long as the desired activity or functional properties (*e.g.*, enzymatic activity, ligand binding, signal transduction, immunogenicity, etc.) of the full-length or fragment are retained. The term also encompasses the coding region of a structural gene and the sequences located adjacent to the coding region on both the 5' and 3' ends for a distance of about 1 kb or more on either end such that the gene corresponds to the length of the full-length mRNA. Sequences located 5' of the coding region and present on the mRNA are referred to as 5' non-translated sequences. Sequences located 3' or downstream of the coding region and present on the mRNA are referred to as 3' non-translated sequences. The term "gene" encompasses both cDNA and genomic forms of a gene. A genomic form or clone of a gene contains the coding region interrupted with non-

population and is thus arbitrarily designed the "normal" or "wild-type" form of the gene. In contrast, the term "modified" or "mutant" refers to a gene or gene product that displays modifications in sequence and or functional properties (*i.e.*, altered characteristics) when compared to the wild-type gene or gene product. It is noted that naturally occurring mutants can be isolated; these are identified by the fact that they have altered characteristics (including altered nucleic acid sequences) when compared to the wild-type gene or gene product.

As used herein, the terms "nucleic acid molecule encoding," "DNA sequence encoding," and "DNA encoding" refer to the order or sequence of deoxyribonucleotides along a strand of deoxyribonucleic acid. The order of these deoxyribonucleotides determines the order of amino acids along the polypeptide (protein) chain. The DNA sequence thus codes for the amino acid sequence.

As used herein, the terms "an oligonucleotide having a nucleotide sequence encoding a gene" and "polynucleotide having a nucleotide sequence encoding a gene," means a nucleic acid sequence comprising the coding region of a gene or in other words the nucleic acid sequence that encodes a gene product. The coding region may be present in a cDNA, genomic DNA or RNA form. When present in a DNA form, the oligonucleotide or polynucleotide may be single-stranded (*i.e.*, the sense strand) or double-stranded. Suitable control elements such as enhancers/promoters, splice junctions, polyadenylation signals, etc. may be placed in close proximity to the coding region of the gene if needed to permit proper initiation of transcription and/or correct processing of the primary RNA transcript. Alternatively, the coding region utilized in the expression vectors of the present invention may contain endogenous enhancers/promoters, splice junctions, intervening sequences, polyadenylation signals, etc. or a combination of both endogenous and exogenous control elements.

As used herein, the terms "complementary" or "complementarity" are used in reference to polynucleotides (*i.e.*, a sequence of nucleotides) related by the base-pairing rules. For example, for the sequence "A-G-T," is complementary to the sequence "T-C-A." Complementarity may be "partial," in which only some of the nucleic acids' bases are matched according to the base pairing rules. Or, there may be "complete" or "total" complementarity between the nucleic acids. The degree of complementarity between nucleic acid strands has significant effects on the efficiency and strength of hybridization between nucleic acid strands. This is of particular importance in amplification reactions, as well as detection methods that depend upon binding between nucleic acids.